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Site-Directed Cross-Linking: A New Approach to Mapping Antibody Combining Sites

(immune/myeloma/3-aminotyrosine/heavy chains/light chains)

NORTIN M. HADLER AND HENRY METZGER

Arthritis and Rheumatism Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT The [illegible] protein 315 from the mouse was affinity-labeled with *m*-nitrobenzene diazonium fluoroborate which, as shown previously, leads to selective modification of the tyrosine at position 34 in the light chains of this protein. The azotyrosine bond was reduced with dithionite to form 3-aminotyrosine. The [illegible] group of the [illegible]tyrosine was selectively reacted with the bifunctional reagent 1,5-difluoro-2,4-dinitrobenzene. Cross-links were formed between the aminotyrosine and at least [illegible] residue—one on the same light chain and one in the [illegible] region of the heavy chain. Since affinity labeling of [illegible] antibodies with diazonium reagents has [illegible] led to azotyrosine formation, the approach [illegible] in this paper should have general applicability.

When the nitrophenyl-binding [illegible] protein 315 of the mouse reacts with low molar ratios of *m*-nitrobenzene diazonium fluoroborate, the tyrosine at position 34 on the light chain is selectively modified [illegible]. The binding sites of [illegible] "affinity-labeled" protein [illegible] are substantially inactivated. Modification is considerably reduced when high concentrations of [illegible] ligands are present during the incubation with the diazonium reagent. Because of these findings, and for other reasons (3), we believe that tyrosine 34 participates in the combining sites of protein 315.

We now report how modification of affinity-labeled tyrosine [illegible] used to further explore the combining site. Our strategy [illegible] follows. Affinity-labeled protein 315 was reduced with dithionite ($Na_2S_2O_4 \cdot 2H_2O$) to yield 3-aminotyrosine (NH_2-Tyr) at position 34 on the light chain. Advantage was taken of the [illegible] low pK of the amino group of NH_2-Tyr to react it selectively with the cross-linking reagents 1,5-difluoro-2,4-dinitrobenzene (F_2DNB) and *p,p'*-difluoro-*m,m'*-dinitrodiphenyl sulfone (F_2DPS). The [illegible] protein was examined for evidence of cross-linking between the NH_2-Tyr residue and other amino acid side chains.

METHODS

Protein 315 was affinity-labeled with [illegible] moles of *m*-nitrobenzene diazonium fluoroborate per mole of protein at pH [illegible] as previously described (2).

Reduction with dithionite was performed at room temperature in a 0.05 M borate [illegible] (pH 9.6) buffer. Usually 1.5–2.0 [illegible] M protein was reduced by adding one-tenth [illegible] volume of 0.15 M dithionite in 0.05 M $NaHCO_3$. After [illegible] the protein was [illegible] dialyzed against [illegible] M Na citrate buffer (pH [illegible]).

Reactions with the cross-linking reagents were performed as described by Cuatrecasas *et al.* (4). Small amounts of an acetone solution of the cross-linking reagent were added every 10 min to [illegible] $\times 10^{-5}$ M protein in 0.5 M Na citrate buffer (pH [illegible]) so that by 45 min a 10-fold molar excess of reagent [illegible] been added. The reaction was allowed to proceed for another 3 hr in the dark. Unbound reagent was removed by chromatography of the mixture on a Sephadex G-25 column equilibrated with a pH 5.2 buffer that was 5 mM in Na citrate and 0.15 M in NaCl. The protein was then diluted to 5×10^{-6} M in 0.1 M $NaHCO_3$ and adjusted to pH 9.4 with 0.1 N NaOH, in order to allow any unreacted fluoride groups to react. After 16 hr at room temperature in the dark, the protein was concentrated and dialyzed against a convenient buffer.

Spectra of the modified proteins were recorded on a Cary [illegible] 15) spectrophotometer. Spectra were obtained between [illegible] and 600 nm at pH 5 and 9.4 both before and after hydrolysis with 1 M NaOH for 2 hr at room temperature. Hydrolysis [illegible] unreacted fluoride groups to yield a nitrophenolate group that has distinctive spectral properties (4). None of the modifications described in this paper significantly changed the absorbance of protein 315 at 280 nm, as shown by sequential determinations during the course of the reaction.

The binding activity of the proteins was assessed by equilibrium dialysis at room temperature in a pH 8.0 borate [illegible] buffer in 0.10-ml lucite chambers. [^{3}H]ϵ-*N*-2,4-dinitrophenyllysine was used as the ligand.

NH_2-Tyr was assayed on a Beckman 120C Autoanalyzer. Protein hydrolysates, prepared in the usual manner, were [illegible] on a 15-cm column at 51°C at pH 4.87 (5).

[illegible] gel electrophoresis was performed in neutral sodium dodecyl sulfate on 7.5% polyacrylamide gels (6) on a Buchler apparatus. Gels were stained with Coomassie brilliant blue. The [illegible] of each band was plotted versus the logarithm of the molecular weight and a line was drawn through the points representing components of known molecular weights. The molecular weight of the unknown components was then [illegible] from their R_f.

MATERIALS

Immunoadsorbent purified protein 315 was prepared from ascites raised in BALB/c mice carrying the MOPC-315 tumor (2). The peritoneal fluid was reduced with dithiothreitol and alkylated with iodoacetamide prior to purification of the

Abbreviations: F_2DNB, 1,5-difluoro-2,4-dinitrobenzene; F_2DPS, *p,p'*-difluoro-*m,m'*-dinitrodiphenyl sulfone; NH_2-Tyr, 3-aminotyrosine.

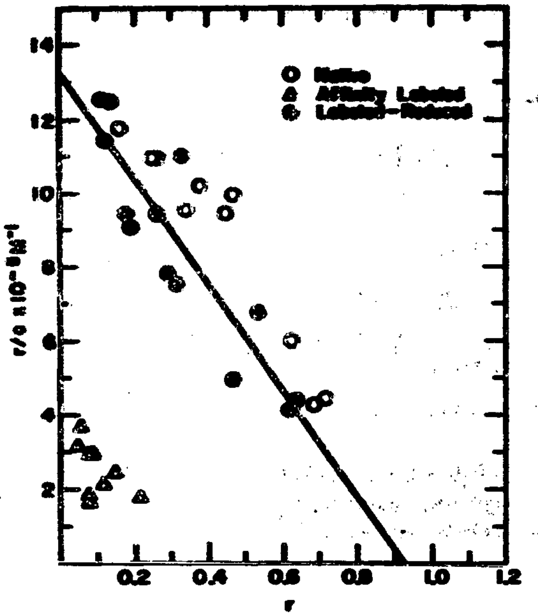

FIG. 1. Scatchard plot of equilibrium dialysis data on native, affinity-labeled, and affinity-labeled, then reduced protein 315. Dialyses performed at room temperature in borate–NaCl buffer (pH 8.0), with [^{3}H]ε-*N*-2,4-dinitrophenyllysine as ligand.

myeloma protein (2). The purified protein has a molecular weight of 150,000 (7); since the interchain disulfides have been cleaved, the polypeptide chains are held together by noncovalent interactions only. The extinction coefficient for the protein is 1.44 for a 0.1% solution (8).

Fab fragments were prepared by digesting protein 315 with 1% (w/w) trypsin at 37°C in 0.2 M Tris·HCl-0.01 M $CaCl_2$ buffer (pH 8.0) for 3 hr. Digestion was stopped with soybean trypsin inhibitor. The Fab fragments were isolated by chromatography on a Sephadex G-100 column equilibrated with pH 8.0 borate–NaCl buffer.

Preparation of [^{3}H]*m*-nitrobenzene diazonium fluoroborate and its nonradioactive analogue has been described (9, 2).

[^{3}H]ε-*N*-2,4-dinitrophenyllysine (1.3 Ci/mmol) was purchased from New England Nuclear. F_2DNB and F_2DPS were purchased from Sigma and Pierce, respectively, and were used without further purification.

Trypsin that had been reacted with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone was purchased from Worthington Biochemicals Corp. Equine heart cytochrome *c* was purchased from Calbiochem. Acrylamide was purchased from Canalco and recrystallized from benzene. NH_2-Tyr, used as a standard for amino-acid analyses, was purchased from Nutritional Biochemicals Corp. Sephadex gels were purchased from Pharmacia.

RESULTS

Effect of reduction with dithionite

When affinity-labeled protein 315 reacted with a 1000-fold molar excess of dithionite, its visible absorbance rapidly diminished. When ^{3}H-labeled reagent was used and the reduced preparation was dialyzed, more than 90% of the counts were recovered in the diffusate. Generation of NH_2-Tyr on the light chains only was documented by amino-acid analysis. *Fig.* 1 shows a comparison between native, affinity-labeled, and affinity-labeled, then reduced protein 315 as

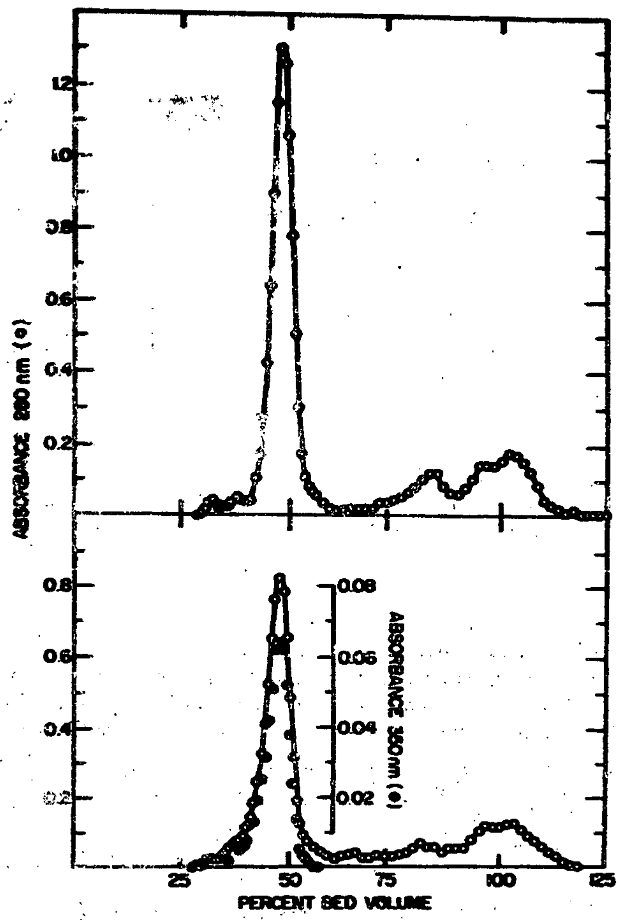

FIG. 2. Elution patterns of Sephadex G-100 chromatography of protein 315 tryptic digests. Columns had a bed volume of 640 ml, were equilibrated with a pH 8.0 borate–NaCl buffer, and were run at room temperature at a flow rate of about 30 ml/hr. *Top*: digest of unmodified protein 315; load about 60 *A* units. *Bottom*: digest of affinity-labeled, reduced, F_2DNB-treated protein 315; load: about 40 *A* units.

studied by equilibrium dialysis. It can be seen that the binding activity that is lost by virtue of the affinity labeling is substantially recovered after reduction with dithionite.

Reaction with F_2DNB

Reaction of F_2DNB with protein can be observed by the change in the protein's absorbance at 350–380 nm. When native protein 315 was incubated with F_2DNB under the conditions used, no reaction was observable.

When affinity-labeled, then reduced protein was used there was a marked change in the spectrum after the incubation at pH 5. However, no further change was observed after the protein was incubated at pH 8.4. At neither stage was there a substantial difference between the spectra recorded at acid and alkaline pH, either before or after alkaline hydrolysis. This result suggested that both fluoride groups had reacted during the initial incubation at pH 5.2 (see *Discussion*).

After the reaction with F_2DNB, little or no binding activity was observable.

Localization of cross-links

Protein 315 that had been affinity-labeled, reduced, and reacted with F_2DNB was subjected to tryptic digestion

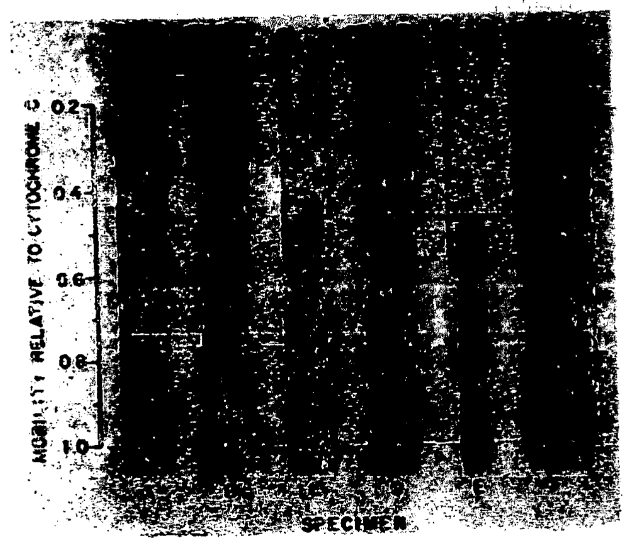

FIG. 3. Disc electrophoresis patterns of protein 315. The most rapidly moving band is bovine cytochrome *c*, which was added to all specimens. Protein loads (exclusive of the cytochrome *c*) were about 40 μg per gel. *A*, Human Bence-Jones protein, λ Riggs, which exists primarily as a disulfide-linked dimer. Only a faint monomer band is seen. *B*, Unmodified protein, 315. *C*, Fab fragments isolated from a tryptic digest of unmodified protein 315. *D*, Affinity-labeled, reduced, F_2DNB-treated protein 315. *E*, Fab fragments isolated from tryptic digest of the preparation shown in *D*. *F*, Preparation treated similarly to that in *D*, except that affinity labeling was substantially prevented (*see text*).

(*Materials*), and the digest was chromatographed on Sephadex G-100. As can be seen in Fig. 2, the nitrophenyl absorbance chromatographed identically with Fab fragments derived from native protein. The Fc fragments were mainly cleaved into smaller peptides under the conditions of digestion employed. This experiment shows that no intermolecular or inter-Fab cross-linking occurred.

Protein 315 that had been affinity-labeled, dithionite-reduced, and reacted with F_2DNB was electrophoresed on detergent–polyacrylamide gels and compared with native protein 315. Fab fragments from both preparations were also compared. In addition, a control specimen was prepared as follows: resorcinol was added to protein 315 prior to the addition of the affinity-labeling reagent so that the amount of affinity labeling would be substantially reduced. This specimen was then handled exactly like the affinity-labeled preparation. All the samples were chromatographed on Sephadex G-200 or G-100, and the main components were isolated prior to analysis. Typical gel patterns are reproduced in Fig. 3.

The noteworthy finding is that the preparations that had reacted with F_2DNB show new, slower components not seen with the native proteins. The molecular weight of these new components was estimated as indicated in *Methods*, using 13,700 for the mol wt of cytochrome *c* and the values for the light and heavy chains shown in Table 1.

It can be seen from the table that the new band in the F_2DNB-treated protein has an apparent molecular weight that corresponds to that expected for a light chain linked to a heavy chain. The new band observed in the Fab fragments of such a preparation had an R_f consistent with that of a light chain linked to an Fd fragment.

The control preparation (*F*) showed only a faint band in the region where a strong band appeared in the cross-linked preparation.

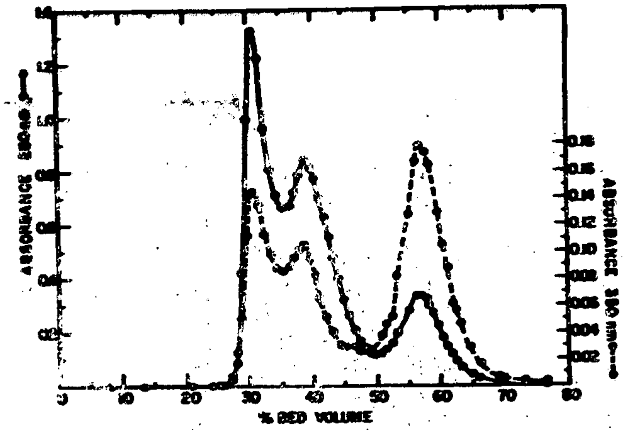

FIG. 4. Elution pattern of Sephadex G-100 chromatography of protein 315 that had been affinity-labeled, reduced, and reacted with F_2DNB. The column had a bed volume of 500 ml, was equilibrated with 6 M urea–0.1 M acetic acid, and was run at about 15 ml/hr. The protein load was about 150 *A* units.

When intact protein (Fig. 4) or tryptic Fab fragments (not shown) were chromatographed on Sephadex G-100 in 6 M urea–0.1 M acetic acid—conditions used to separate heavy and light chains—substantial absorbance due to the dinitrophenyl group was observed under the peak containing light chains, as well as in the region where the cross-linked heavy chain–light chain complex eluted. The light-chain peak contained 18% of the absorbance at 280 nm and 48% of the absorbance at 350 nm. Protein 315 has in past experiments given patterns in which the light-chain peak represented 26% of the absorbance at 280 nm (1). Thus, about 30% of the light chains are incorporated in the heavy-chain peaks. Since these light chains, putatively cross-linked to heavy chains, must account for the 52% of the absorbance at 350 nm seen in the heavy-chain peaks, it can be calculated that (very roughly) 0.5–0.6 mol of F_2DNB was incorporated into the protein. That the biphasic heavy-chain peak results from aggregation and not from a resolution of cross-linked heavy–light chains (from heavy chains) is suggested by two considerations: (*a*) since only the light chains contain NH_2-Tyr, F_2DNB would not be expected to have reacted with the heavy chains by themselves, and (*b*) samples taken throughout the heavy-chain peak showed a dark, slow band, equivalent to

TABLE 1. *Molecular weights estimated from disc electrophoresis*

Component	R_f	Molecular weight
315 light chain	0.79	23,000*
315 heavy chain	0.51	52,000*
Fd fragment	0.68	32,500†
Fd fragment–light chain	0.50	56,000†
		55,000‡
Heavy chain–light chain	0.38	79,000†
		75,000‡

* Assumed.
† Estimated from graph (see text).
‡ Expected.

the band expected for cross-linked heavy-light chains, on disc electrophoresis. These observations suggest that in addition to a cross-link between NH_2-Tyr and a heavy-chain residue, a cross-link between NH_2-Tyr and another light-chain residue had occurred.

Reaction with F_2DPS

The affinity-labeled, dithionite-reduced protein reacted smoothly with F_2DPS at pH 5. After removal of unreacted reagent by Sephadex G-25 chromatography, a covalently bound chromophore could be generated after 2 hr of hydrolysis in 1 N NaOH at room temperature; this indicates the presence of a free fluoride group. Even after 7 days of incubation at pH 9.4, this second fluoride group had failed to react!

DISCUSSION

The strategy for exploring the combining sites of myeloma protein 315 described in this paper was based on the study by Cuatrecasas *et al.* (4). These authors nitrated various functional forms of staphylococcal nuclease, reduced the nitrated tyrosines with dithionite, and reacted the NH_2-Tyr groups thereby generated with F_2DNB and F_2DPS. Because of the unusually low pK of the aryl amino group in NH_2-Tyr [pK = 4.7 (5)], the cross-linking reagents reacted at pH 5 with these groups in preference to all other nucleophilic constituents. Since we had previously shown that tyrosine-34 on the light polypeptide chains of protein 315 could be selectively modified by the affinity-labeling reagent, and since it is known that phenylazo bonds can be reduced with dithionite to give aniline derivatives (10), the experiments described here seemed feasible.

Our results differ somewhat from the findings of Cuatrecasas *et al.* (4). In their studies, the first fluoride group in F_2DNB and F_2DPS reacted at pH 5 but the second one did not. This was demonstrated by hydrolysis of the pH 5 derivative with 1 N NaOH. The second fluoride was removed to yield the dinitrophenolate ion, and observable spectral changes were thereby produced. In the nuclease studies, it was necessary to reincubate the pH 5 derivatives at pH 9.4 in order to complete the cross-link by reaction of the second fluoride group with epsilon-amino groups of lysines. In our own studies, a similar phenomenon was observed with F_2DPS. However, even after prolonged incubation at pH 9.4, no evidence was obtained that the second fluoride had reacted. With F_2DNB, on the other hand, we could never isolate the two steps involved in the cross-linking reaction—the reaction with NH_2-Tyr was followed immediately by the reaction of the second fluoride with another, as yet unidentified, nucleophile. We are uncertain why this happened. It is possible that our use of a dinitrophenyl cross-linking reagent on a nitrophenyl binding site is significant. Studies with nonnitrophenyl cross-linking reagents with protein 315, and with F_2DNB with combining sites of other specificities on immunoglobulins will be required to clarify this point. It would not be surprising if the phenomenon we observed were found fairly frequently. The reaction of the second fluoride is a first-order process whose rate should be sensitive to the spatial relationship of the fluoride group and an available nucleophile. With favorable spatial relationships, the rate of reaction could be extremely rapid even at nominally unfavorable pH values.

Cross-linking of the light-chain tyrosine to another nucleophile could have led to a bridge between light-chain or heavy-chain amino-acid side chains. These residues could have been on the same Fab as the NH_2-Tyr, on the alternate Fab within the same molecule, or on a Fab from a second molecule. The latter two alternatives were ruled out by our finding that the nitrophenyl cross-links were present on monomeric Fab fragments (Fig. 2). The experiment illustrated in Fig. 4, and a comparable study with Fab fragments, showed that two types of cross-links occurred—one or more between NH_2Tyr and another light-chain residue, and one or more between NH_2-Tyr and a Fd-region residue.

These results add further evidence for, and indeed provide some of the most explicit information about, the intimate relationship between heavy- and light-chain amino-acid side chains in immunoglobulin-combining sites. The maximum distance between the 3-amino group of NH_2-Tyr at position 34 on the light chain and the residues on the light and heavy chains to which it was cross-linked by F_2DNB is about 6 Å (11). It will be of considerable interest to see if the cross-linked residues fall within the hypervariable regions of the light and heavy chains—regions that are becoming increasingly implicated as participating in the antibody-combining site (3, 12, 13).

We wish to emphasize some general features of our study. Diazonium reagents have been generally useful as affinity-labeling reagents for various antibodies of different specificities from several different species. With only rare exceptions, the residue that is specifically modified is a light-chain or heavy-chain tyrosine (14, 15). With most phenyl diazonium compounds likely to be used, the reduction of the azotyrosine bond with dithionite should be possible. Thus, it should be feasible in many instances to generate NH_2-Tyr in the combining sites of antibodies. By judicious selection of various cross-linking reagents it should, therefore, be possible to explore several antibody-combining sites by the procedures described in this paper.

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